2-Dimethylaminoethyl 4-(o-chlorobenzyl)-4-phenyl-5-oxohexanoate hydrobromide. 4-(*o*-Chlorobenzyl)-4-phenyl-5-hydroxy-5-hexenoic acid lactone (36.1 g., 0.115 mole) was dissolved in dry 2-dimethylaminoethanol (89 g., 1 mole) and the mixture was heated at 95° under anhydrous conditions. After a few minutes at this temperature the mixture was refluxed for 2 hr. The excess amine was removed by distillation at reduced pressure and the residue was dissolved in benzene (250 ml.).

The solution was washed with water twice and then dried over sodium sulfate. The benzene was removed by distillation and the residue dissolved in cyclohexane (150 ml.) and dry hydrogen bromide was introduced into the solution until precipitation no longer occurred. Addition of benzene (50 ml.) caused the conversion of the gummy product to a white solid, 48.2 g. (87%), m.p. 144–148°. Two recrystallizations from isopropyl alcohol gave 41.7 g., m.p. 149–151°.

Anal. Calcd. for $C_{23}H_{29}BrClNO_3$: C, 57.20; H, 6.05; N, 2.90; Br, 16.55. Found: C, 56.95; H, 6.35; N, 2.90; Br, 16.59.

levo-2-Dimethylaminoethyl 4-(o-chlorobenzyl)-4-phenyl-5-oxohexanoate hydrobromide. levo-4-(*o*-Chlorobenzyl)-4-phenyl-5-oxohexanoic acid lactone (37.5 g., 0.12 mole) and freshly distilled dry 2-dimethylaminoethanol (107 g., 1.2 moles) were refluxed for 2 hr. and the product isolated as described for the racemic modification. The free base was dissolved in isopropyl alcohol and treated with hydrogen bromide gas to give 52.6 g. (90%) of the hydrobromide salt. Two recrystallizations from isopropyl alcohol gave 46.5 g. of pure material, m.p. 149.5–151.5°. The $[\alpha]_D^{26}$ of a 1% water solution was −37.7°.

Anal. Calcd. for $C_{23}H_{29}BrClNO_3$: C, 57.20; H, 6.05; N, 2.90; Br, 16.55. Found: C, 57.65; H, 5.98; N, 2.86; Br, 16.49.

4-(o-Chlorobenzyl)-4-phenyl-5-oxo-7-dimethylaminoheptanoic acid hydrochloride. 4-(*o*-Chlorobenzyl)-4-phenyl-5-oxohexanoic acid (66.2 g., 0.2 mole), dimethylamine hydrochloride (16.3 g., 0.2 mole), and paraformaldehyde (6.6 g., 0.22 mole) were intimately mixed and placed in a one-liter flask fitted with a Claisen head and receiver. The system was evacuated by means of a water aspirator and the mixture heated at 140° using a metal bath.

The solids melted and vigorous bubbling occurred for a few minutes. After heating at 135–145° for 30 min. the mixture was cooled and the resulting sirup was digested with butanone. After cooling the solid that had formed was removed by filtration and dried. The yield was 48.6 g. (57%), m.p. 123–139°. Two recrystallizations from isopropyl alcohol gave 29.2 g. of pure material, m.p. 165–167°.

Anal. Calcd. for $C_{22}H_{27}Cl_2NO_3$: C, 62.26; H, 6.41; N, 3.30; Cl, 16.69. Found: C, 62.73; H, 6.17; N, 3.25; Cl, 16.93.

levo-4-(o-Chlorobenzyl)-4-phenyl-5-oxohexanamide. Ammonia gas (31 g., 1.8 moles) was dissolved in 2-methoxyethanol (200 ml.) at −40°. *levo*-Methyl 4-(*o*-chlorobenzyl)-4-phenyl-5-oxohexanoate (25 g., 0.073 mole) was dissolved in 2-methoxyethanol (50 ml.) and the solution cooled to −40°. The two solutions were united in an autoclave and heated at 100° for 4 hr. After standing at room temperature for 48 hr. the solvent was removed by distillation at reduced pressure.

The residue was dissolved in benzene (150 ml.) and washed with saturated sodium bicarbonate solution (25 ml.) and then with water (25 ml.). The benzene solution was dried over sodium sulfate and the solvent was removed by distillation. Treatment of the residue with a mixture of benzene and hexane gave a solid. The yield was 14.7 g. (64%), m.p. 58–69°. After one recrystallization from a mixture of benzene and hexane and two recrystallizations from benzene, 8.1 g. remained, m.p. 81–83°.

Anal. Calcd. for $C_{19}H_{20}ClNO_2$: C, 69.19; H, 6.11; N, 4.25; Cl, 10.75. Found: C, 69.97; H, 6.22; N, 4.16; Cl, 10.38.

4-(m-Carboxyphenyl)-3-phenyl-2-butanone. 4-(*m*-Cyanophenyl-3-phenyl-2-butanone (25 g., 0.1 mole) was suspended in a mixture of glacial acetic acid (150 ml.), water (70 ml.), and concd. sulfuric acid (50 ml.). The mixture was refluxed for 2 hr. and then cooled and poured into water (2 l.). The oil that separated was extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness and the residue treated with an excess of aqueous potassium hydroxide solution. The solution was extracted with a little benzene to remove a small amount of insoluble material.

The aqueous solution was acidified with hydrochloric acid. The oil which separated initially solidified upon standing. The product was removed by filtration and dried. The yield was 18.6 g. (70%), m.p. 86–90°. Two recrystallizations from cyclohexane gave pure material (15.1 g.), m.p. 87–89°.

Anal. Calcd. for $C_{17}H_{16}O_3$: C, 76.10; H, 6.01. Found: C, 76.35; H, 6.01.

WEST POINT, PA.

[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Further Studies with the Bacitracin Polypeptides*

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The fractionation of bacitracin by countercurrent distribution has been reinvestigated from the standpoint of obtaining a product with the highest antibiotic activity. Evidence has been given that the decrease in antibiotic activity at lower *p*H may be associated with a change in the optical activity of at least one of the amino acid residues, probably the isoleucine residue forming part of the thiazoline ring. This is a transformation apparently not related to that occurring at higher *p*H which leads to the F type of peptide.

Preliminary data for the characterization of bacitracin B have been given. These include amino acid analyses, ultimate analyses, determination of the amino groups capable of reacting with the Sanger reagent, and antibiotic activity.

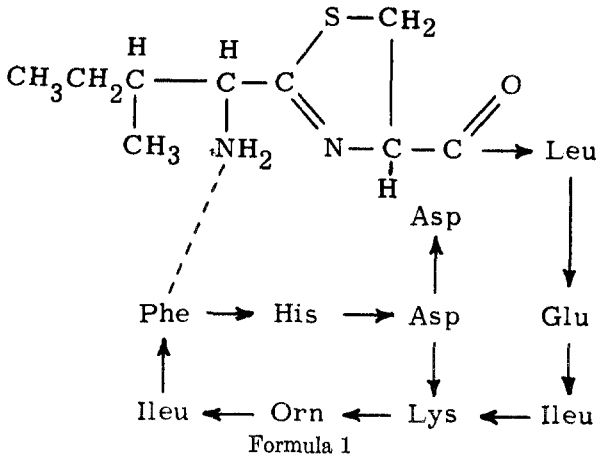
The bacitracin polypeptides[1] comprise an interesting family of naturally occurring antibiotics with unique structures. None of them has been brought to a crystalline state as yet, although the major features of a structural formula for bacitracin A, the most abundant member of the family, appear to have been established. Formula 1 derived in this laboratory[1,2] and independently in England by

* This paper is a contribution in honor of Lyndon F. Small, former Editor of the Journal.

** Fellow of the National Science Foundation.

(1) L. C. Craig, 3rd Congr. Intern. Biochim., Brussels, 1955, 416.

(2) J. R. Weisiger, W. Hausmann, and L. C. Craig, *J. Am. Chem. Soc.*, 77, 3123 (1955).



Formula 1

Lockhart, Abraham, and Newton[3] rationalizes nearly all the experimental observations thus far made. Although an earlier preliminary communication by Porath[4] suggested an amino acid sequence which bore little relationship to Formula 1, a later paper[5] has stated that the data accumulated in this third laboratory which bears on the structure of bacitracin A, are now in agreement with those from the other two laboratories.

Formula 1, therefore, can be regarded as a provisional working formula but there remains still a number of puzzling observations to be substantiated and explained.

One of the uncertain features involves the quantitative antibiotic activity of bacitracin A. The standard preparation of A prepared in this laboratory by countercurrent distribution (C.C.D.) with the system 2-butanol/3% acetic acid always gave an assay approximating 50 units per mg. This material has been shown to be the acetate[6] and in addition contains 10–15% of solvent which can be removed at 100°. Even correcting for the acetate and the solvent the material appeared to be less active than the commercial product. This discrepancy was made more apparent by the fact that C.C.D. and ultraviolet absorption spectrum measurements always showed the commercial preparation to contain about 20% of the F type of peptide, a transformation product with very low activity. A number of reports in the literature[7,8] have given activities above 70 u/mg. A background such as this clearly suggests that further fractionation work is needed.

Assuming the correctness of the thiazoline ring system in Formula 1, it seemed that this might offer a clue to the difficulty of obtaining a product with maximum activity, to say nothing of preserving the activity level after it had been achieved. Such a thiazoline offers a very labile structure as is known for other synthetic thiazolines.[9] Thus the double bond might not always keep the position shown in the formula and might be expected to cause racemization of the alpha carbon atom of the isoleucine residue. Racemization of the single center of asymmetry could reduce the antibiotic activity of a preparation to half but would be expected to have very little effect on the physical properties of the molecule such as the partition ratio. A mixture of diastereoisomers of the size of bacitracin could well behave as a pure substance by C.C.D.

With the thought that the *p*H of the solvent might have an influence on the more subtle changes of the type mentioned, the fractionation of commercial bacitracin by C.C.D. has again been taken up. Newton and Abraham[10] used a system buffered with phosphate at *p*H 7, but in our hands their system gave rise to a slow but steady transformation[11] of bacitracin A to F. The low *p*H of the 3% acetic acid system completely inhibited the A to F transformation, but could have accelerated another type of transformation with loss of activity. In order to test this hypothesis several new systems offering a variety of *p*H levels have been developed. It also appeared worthwhile to investigate the effect of other solvents on the stability.

It seemed that this approach could well be combined with a careful study of optical rotation of the fractions. Although earlier measurements of optical activity with bacitracin A had shown very little, if any, rotation, reinvestigation at higher concentration could well be rewarding. DNP amino acids usually show higher rotations than the free amino acids. A study has therefore been made of the optical activity of the various fractions obtained when bacitracin A and B are treated with fluoro-2, 4-dinitro benzene. This study could also serve to confirm or cast doubt on the thesis of purity.

In a family of closely related natural products valuable information is usually derived by the simultaneous study of several members of the family. With the bacitracins very little has been reported thus far concerning the nature of B, C, D, etc., although F has been shown to be a transformation product[2,10] of A. In the course of the fractionation studies presented in this paper a moderate supply of bacitracin B has been accumulated. A number of preliminary observations regarding its relationship to A can now be reported.

(3) I. M. Lockhart, E. P. Abraham, and G. G. F. Newton, *Biochem. J.*, **61**, 534 (1955).
(4) J. Porath, *Nature*, **172**, 871 (1953).
(5) J. Porath, *Acta Chem. Scand.*, **8**, 1813 (1954).
(6) W. Hausmann, J. R. Weisiger, and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 721 (1955).
(7) I. M. Lockhart, G. G. F. Newton, and E. P. Abraham, *Nature*, **173**, 536 (1954).
(8) M. G. Gollaher and E. J. Honohan, U. S. Patent **2,763,590** (To Chas. Pfizer and Co.).
(9) K. Linderstrom-Lang and C. F. Jacobsen, *J. Biol. Chem.*, **137**, 443 (1941).
(10) G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **53**, 597 (1953).
(11) L. C. Craig, J. R. Weisiger, W. Hausmann, and E. J. Harfenist, *J. Biol. Chem.*, **199**, 259 (1952).

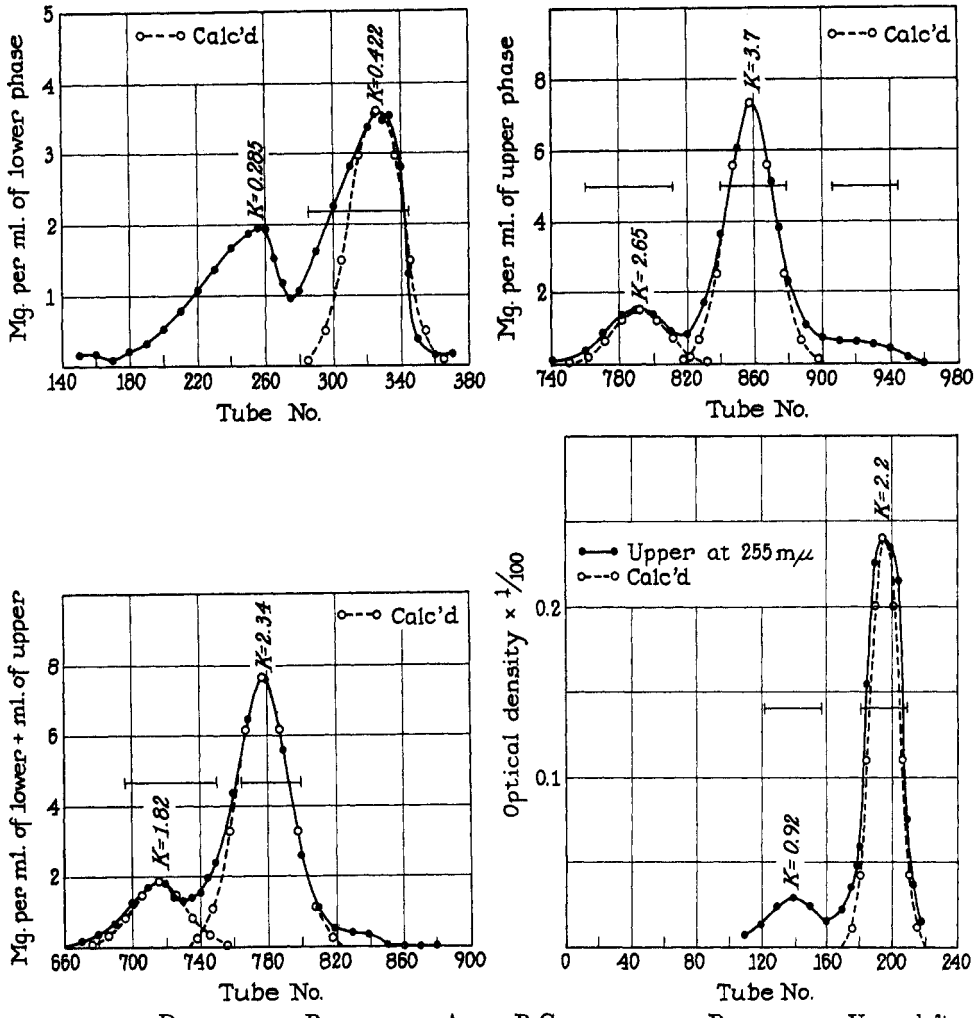


FIG. 1. COUNTERCURRENT DISTRIBUTION PATTERNS OF A AND B COMPONENTS OF BACITRACIN. Upper left; pattern 1. Upper right; pattern 2. Lower left; pattern 3. Lower right; pattern 4.

EXPERIMENTAL

The bacitracin used in the present study was obtained from the Commercial Solvents Corporation. We wish to express our thanks to them for this material and for all the antibiotic assays reported in this paper.

The distributions were made at 25° in an automatic C.C.D. apparatus of the type described earlier.[12] All solvents were distilled prior to use.

Our experience with four systems in fractionating bacitracin will be reported. These systems were the following:

1. 1-butanol, glacial acetic acid, and water in the volume proportions of 75, 15, 100 respectively.
2. 1-butanol, glacial acetic acid, pyridine, and water in the proportions of 20, 5, 5, 30. *p*H of lower phase: 4.59.
3. 1-butanol, pyridine, 0.1% acetic acid in the volume proportions of 15, 9, 33. *p*H of lower phase: 6.7.
4. 1-butanol, 0.5 *M* phosphate buffer at pH 5.4 in equal volumes.

In each system the initial load of peptide was 5 g., scattered in the first 10 tubes of the train. Sufficient amount of the lower phase was added at each transfer during the run to maintain the level of the lower phases. The settling time for the first three systems approximated 1 min., but about 5 min. for the fourth.

Analyses in the first three runs were made by weight, but in the fourth by optical density at 255 mμ since it contained phosphate buffer. For the weight analysis in the first run, 1 ml. of the lower phase was evaporated, dried at 100°, and weighed. In the second, 1 ml. of the upper phase was evaporated, while in the third, 1 ml. of lower phase plus 1 ml. of upper phase were evaporated.

The distribution patterns for the A and B components in each of the four runs is shown in Fig. 1. The C component in each run was removed before the run was completed and is not shown. Thus in run 1 at 220 transfers, effluent was collected in the fraction collector, but at 280 transfers all the F had emerged from the train. It was then arranged for recycling. At 580 transfer the A, B, and C components were apparent with the C component well separated with a maxima at tube 67 (K = 0.132) and amounting to about one third of the B component. This is more of the C component than appeared in the following runs. Run 1 was made on a preparation (Lot B-277B) received a year or more earlier than that (Lot B-55-10) on which the others were made. At 580 transfers the C component was removed and the distribution continued to 1098 transfers to give pattern 1.

In the case of run 2, the F component was removed at 560 transfers. It had a K of 9.5 in this system. The distribution was then continued to 1090 transfers to give pattern 2. Very little of the C component was found, but some of it may be in the left hand side of the B band as an overlap.

With run 3, the F component was removed at 656 transfers. It had a K of 3.8 in this system. At 1110 transfers pattern 3 was obtained.

Since the selectivities of the system were higher in run 4, it was stopped at 292 transfers. The F component which

(12) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, 23, 1236 (1951).

had a K of 7 was permitted to emerge from the train as effluent. At the beginning some difficulty was experienced in the separation of the phases, but soon a settling time of 5 min. was possible.

For recovery, cuts were taken as shown in Fig. 1. With the first three systems the organic solvents were removed by evaporation in the rotary evaporator[13] until an aqueous solution remained. This was freeze-dried.

With the fourth run the cuts were treated with 60 ml. of $2M$ K_2HPO_4 and shaken. The butanol phase containing the peptide was set aside and the aqueous phase extracted with fresh 1-butanol. The butanol extracts were treated with water and the butanol removed in the rotary evaporator. The aqueous solutions remaining were freeze-dried. This material contained considerable phosphate. The phosphate was removed by a 30-transfer distribution of each cut in system 3. It had a low K in this system and was easily removed.

When an attempt was made to obtain reproducible analytical results on the recovered bacitracins A and B, difficulty was encountered in choosing conditions for drying the sample. This was also complicated by the fact that the dry sample was hygroscopic and tended to fly out of the boat due to static electricity. Drying in high vacuum to constant weight at 100° was finally chosen. The recovered A from run 1 gave analytical data in agreement with that previously published[6] for the acetate but in the case of the other three systems the final product was recovered by freeze-drying a pyridine acetate solution and gave figures more in agreement with the free peptide.

Anal. Calcd. for $C_{66}H_{102}O_{16}N_{17}S$: C, 55.6; H, 7.3; N, 16.7. Found: C, 55.4; H, 7.3; N, 16.8.

In the case of bacitracin B the material from run 1 should be the acetate.

Anal. Calcd. for $C_{73}H_{116}O_{19}N_{18}S$: C, 55.3; H, 7.4; N, 15.9; S, 2.1. Calcd. for $C_{73}H_{118}O_{20}N_{18}S$: C, 54.8; H, 7.4; N, 15.7; S, 2.0. Found: C, 54.4; H, 7.4; N, 15.5; S, 2.3.

With the other three runs as with A, a higher carbon and nitrogen value was obtained indicating the free peptide.

Anal. Calcd. for $C_{71}H_{112}O_{17}N_{18}S$: C, 56.0; H, 7.4; N, 16.5. Calcd. for $C_{71}H_{114}O_{18}N_{18}S$: C, 55.4; H, 7.5; N, 16.4. Found: C, 55.2; H, 7.4; N, 16.4.

A quantitative amino acid analysis of a sample of B from run 4 by the ion exchange method of Moore and Stein[14] was made. This gave the result shown in Table I. The result previously published[2] for bacitracin A is given for comparison. The fraction to be analyzed was directly weighed for hydrolysis and then the weight corrected to dry weight at 100°. Hydrolysis was accomplished by heating in $6N$ HCl at 108° for 24 hr. in a sealed evacuated tube.

TABLE I

AMINO ACID ANALYSES

Amino Acid	Bacitracin B Corr. μM	Bacitracin B Nearest integer	Bacitracin A Corr. μM
Aspartic acid	1.76	2	1.77
Cystine (half)	.812	1	1.0
Valine	.900	1	none
Allo-isoleucine	.330		0.5
Isoleucine	1.89	2	1.83
Leucine	1.02	1	1.02
Phenylalanine	1.08	1	1.03
Lysine + ornithine	2.24	2	1.69
Histidine	1.22	1	0.80
NH_3	1.21	1	1.3

(13) L. C. Craig, J. D. Gregory, and W. Hausmann, *Anal. Chem.*, 22, 1462 (1950).

(14) S. Moore and W. H. Stein, *J. Biol. Chem.*, 211, 907 (1954).

The antibiotic assays were made by the Commercial Solvents Corporation. All assays reported in this paper are corrected to a dry weight (at 100°) basis. Optical rotations were determined in a 5% solution in glacial acetic acid of the peptide with the Keston polarimetric attachment for the Beckmann U.V. spectrophotometer. The setting of the instrument was standardized against sucrose solutions. The antibiotic activities and rotations are given in Table II.

TABLE II

ANTIBIOTIC ACTIVITIES AND OPTICAL ROTATIONS

System	Units/mg. A	Units/mg. B	Units/mg. (2 yrs. later) A	Units/mg. (2 yrs. later) B	$[\alpha]_D^{25}$ (C = 5.0 in 0.01 HAc) A	$[\alpha]_D^{25}$ (C = 5.0 in 0.01 HAc) B
2-Butanol/3% HAc Run 1	58	..	55	..	−3.6	
1-Butanol/HAc, H_2O Run 2	56	50	..	35	...	
1-Butanol/pyridine acetate pH 4.59 Run 3	60	57	51	42	−3.8	
1-Butanol/pyridine acetate pH 6.7 Run 4	76	80	72	70	−2.4	−2.8
1-Butanol/phosphate pH 5.4	72	69	70	65	−2.4	

Tri-DNP-bacitracin B. Fifty mg. of bacitracin B was converted to the tri-DNP derivative as was described for bacitracin A.[2] It was separated from the other reaction products by a 52-transfer distribution in the benzene-chloroform-acetic acid-.01 HCl (1:1:2:1) used for A but with 5/10 upper/lower volumes. The main peak had a partition ratio of 4 in this system as compared to a value of 2.66 in the case of tri-DNP-bacitracin A. It showed a type of absorption spectrum indistinguishable from A. A solution containing 1.143 mg./ml. in acetic acid of the material recovered from the main band showed an optical density of 5.60 at 350 mμ. From these data and from similar data with DNP-isoleucine and di-DNP-histidine indicating molecular extinctions of 14,500 for each, a molecular weight of 1940 can be calculated. Theoretical: 2037.

Hydrolysis of Tri-DNP-bacitracin B. A sample weighing 50 mg. was hydrolyzed in 10 ml. of 1:1 glacial acetic acid-concentrated hydrochloric acid in an evacuated sealed tube at 108° for 24 hr. The acid was evaporated, the residue taken up in a little water and extracted with ethyl ether. Evaporation of the ether gave a yellow residue weighing 7 mg. This was distributed to 120 transfers in a hand-operated 120-tube machine requiring 2 ml. lower phase volumes. The machine permitted up to 4 ml. of upper phase but 1 ml. was used. The system was the benzene, acetic acid, 0.1 N HCl (2:2:1) system[15] previously used with DNP amino acids.

Analysis by optical density at 350 mμ showed two major, partly overlapping bands whose widths were in accord with calculated bands. One had a partition ratio of 2.7 to 2.8 and the other had a partition ratio of 1.5. These correspond to the partition ratios of the DNP derivatives of isoleucine and valine. The yields calculated on the basis of a tri-DNP-derivative were 36% and 33% respectively.

In the distribution of the DNP amino acids from the hydrolysis there was a smaller, well separated band nearer the origin which had a partition ratio suggesting that it could be dinitro aniline with a K in this system of .43.

(15) W. Hausmann, J. R. Weisiger, and L. C. Craig, *J. Am. Chem. Soc.*, 77, 723 (1955).

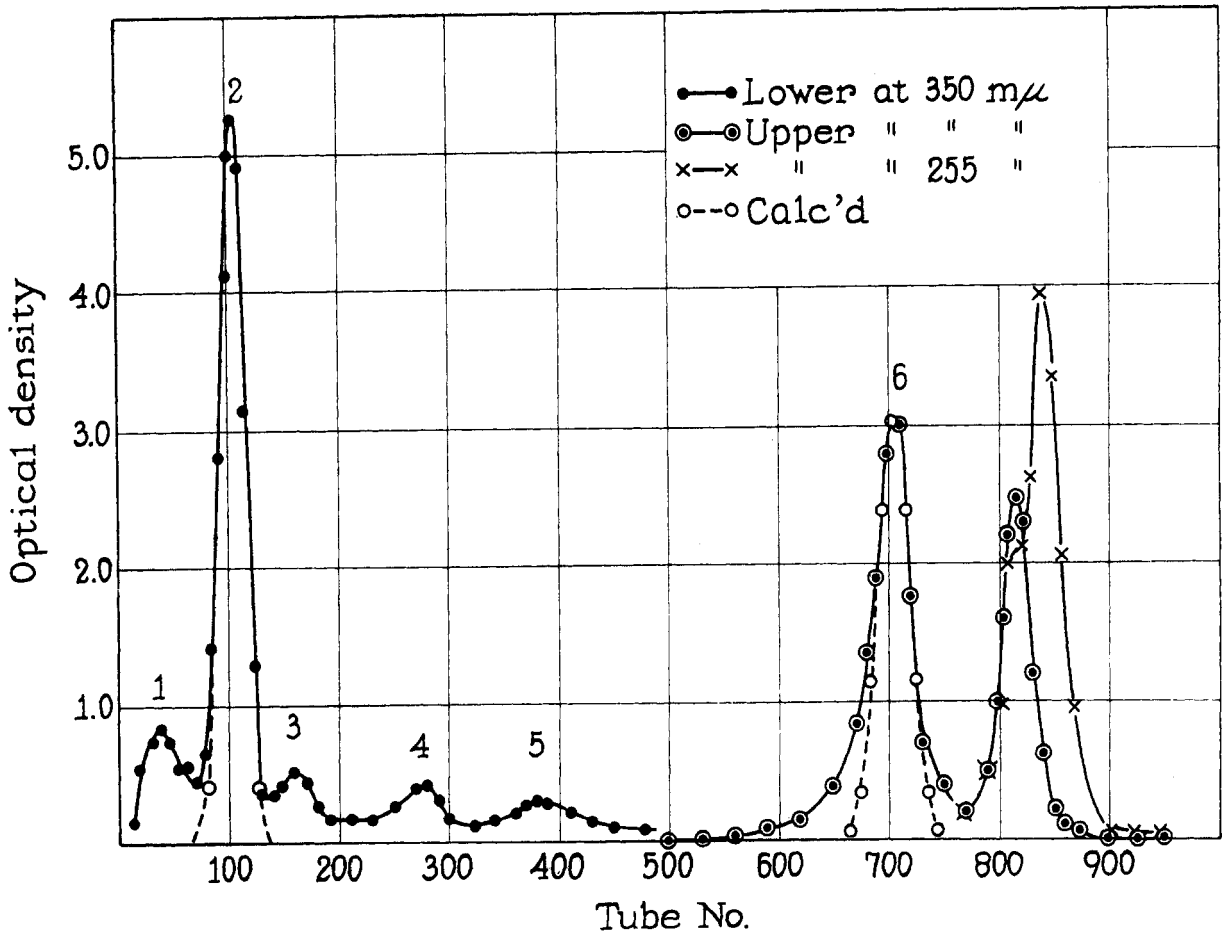


FIG. 2. COUNTERCURRENT DISTRIBUTION PATTERN of the reaction products of bacitracin B with FDNB at *p*H 4.66.

Reaction of fluorodinitrobenzene with the bacitracins at pH 4.66. A sample of the bacitracin weighing 300 mg. was dissolved in 4.5 ml. of 2*M* sodium acetate buffer at *p*H 4.66 and 1.5 ml. of ethanol added. After adding 1.2 ml. of FDNB the mixture was held at 40° for 1 hr. with occasional shaking.

Ethyl ether was added and the ether layer was removed. The suspension remaining was extracted twice more with ethyl ether. Sufficient glacial acetic acid was added to the aqueous suspension to make it a 75% solution with respect to the acetic acid. This solution was dialyzed[16] in 18/32 Visking against 75% acetic acid for 24 hr. This would be expected to remove all the unreacted bacitracin, dinitrophenol, etc.

Sufficient water, ethyl acetate, and chloroform were added to the acetic acid solution so that the solvent composition would approximate that of the system next to be used for the distribution. This system contained chloroform, acetic acid, ethyl acetate, and water in the volume proportions of 2:2:0.2:1. After 1000 transfers analysis by optical density at 350 and 255 mμ gave the pattern shown in Fig. 2 in the case of bacitracin B. This distribution was made in a 1000-tube automatic apparatus with 2 ml. of each phase in every tube.

A cut including the solutes in tubes 775 to 850 was recovered by evaporation of the solvents in a rotary evaporator and redistributed in a more favorable solvent system. This system contained ethyl acetate, glacial acetic acid, water, and 1-butanol in the volume proportions of 3:1:3:0.3 respectively. The apparatus was a hand-operated train of 120 tubes with 2 ml. of each phase per tube. The result after 120 transfers is shown in Fig. 3.

(16) L. C. Craig and T. P. King, *J. Am. Chem. Soc.*, 77, 6620 (1955).

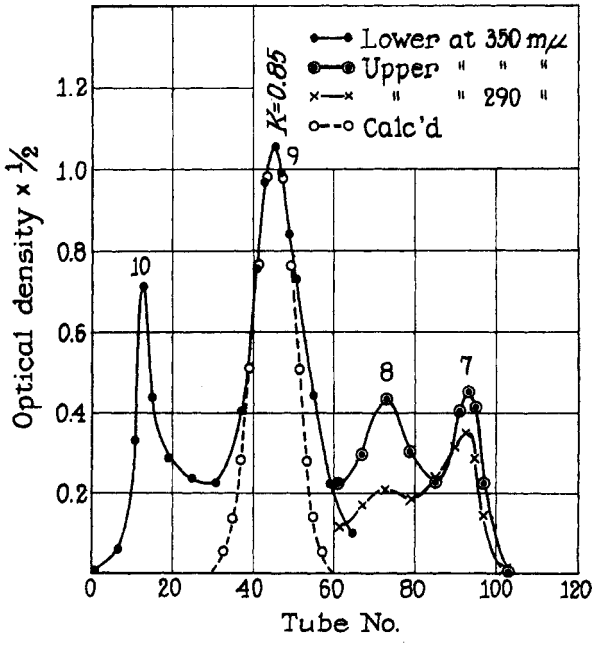


FIG. 3. PATTERN OBTAINED ON REDISTRIBUTING most polar overlapping bands from the distribution shown in Fig. 2.

Conversion of Bacitracin B to the F type. Bacitracin B was found to have an absorption spectrum in the ultraviolet which was almost superimposable on that of A[2] except that its extinction coefficient at 255 mμ was slightly lower.

One hundred milligrams of the peptide was dissolved in 10 ml. of 0.5 *M* phosphate buffer at *p*H 7.59. It showed the same absorption curve as at a lower *p*H. The solution was kept at 38° for two weeks redetermining the absorption curve from time to time. The shallow maximum at 255 slowly decreased but absorption increased in the 290 mμ range. After two weeks there was not even an inflection at 255 mμ but a definite maximum at 290 mμ where F from A has its maximum.

A control experiment with bacitracin A under the same conditions underwent the same type of spectral shift but not as rapidly as did B. This would indicate A to be somewhat more stable than B. The transformation product with B was purified by C.C.D. as reported[2] for the F from A. It had a similar partition ratio. The purified material had an absorption spectrum not different from the crude reaction product and almost superimposable on the spectrum[2] of F prepared from A.

A sample of the F from B was hydrolyzed in 6 *N* HCl in a sealed tube at 108° for 24 hr. The recovered amino acids were studied by two dimensional paper chromatography[15] in the 2-butanol-ammonia system and in the 2-butanol-formic acid system. All the spots found in F from A were present and in addition there was a strong spot in the position of valine.

DISCUSSION

The data given in this paper and shown in Fig. 1 definitely permit a better fractionation by C.C.D. of the bacitracin polypeptides. The exact choice of a system would depend somewhat on the equipment available. Thus with automatic equipment and a long train, system 3 is perhaps best. About the same resolution can be obtained with system 4 in approximately one fourth the number of transfers. It does not permit weight analysis but does permit analysis by optical density. System 3 does not permit analysis by optical density but does by weight. The two systems thus compliment each other very well. With system 4 recovery of the solute in phosphate-free form required a further short distribution in system 3.

Aside from the question of separation, systems 3 and 4 appeared best from the standpoint of recovery of a product with the highest antibiotic activity. The activities shown in the first two columns of Table II were determined a few weeks after the material was recovered. Since the different fractions are freeze-dried preparations they contain varying amounts of solvent up to 15% which can be removed on drying at 100°. A previous experiment with the acetate indicated that the antibiotic potency was not markedly decreased with this treatment. For comparison all the activities reported in this paper are given on the basis of the dry weight. The C.S.C. sample, Lot B-55-10, from which the fractionations were made assayed 67 u/mg. on a similar basis.

After these results were obtained the samples were set aside to await further work. They were stored part of the time in a cold room at +4° and part of the time in a deep freeze at −10°. After about 6 months the A and B fractions from run 3 together with a sample of the C.S.C. starting preparation for control were again sent for assay. A result corresponding to 73, 69, and 65 u/mg., respectively, was obtained. Approximately 18 months later the fractions in Table II were again sent for assay. This gave the results shown in the third and fourth columns. At the time, the control sample of C.S.C., Lot B-55-10, again gave an assay of 65 units.

It had been known for some time[17] that there was a loss of antibiotic potency on recovery of peak material from a distribution in the 2-butanol/3% HAc system since an assay of the solutions directly gave a higher result than after isolation. At first it was thought that this loss was connected with F formation. However, ultraviolet absorption spectral studies as well as redistribution showed F type to be absent from the recovered product. A more subtle type of transformation was indicated.

Optical rotation measurements as a help in this problem at first did not appear promising because of the low rotations found. Four of the amino acid residues emerge after acid hydrolysis as predominantly dextrorotary aside from the N terminal isoleucine. However, a careful study in 5% solution has now given significant results as shown in Table II. These measurements were made on material stored for two years. The more highly potent preparations were found to have a lower optical activity. This was further confirmed by measuring the optical activity at other *p*H values.

It was hoped that direct evidence might be obtained that the terminal isoleucine is involved in the shift of rotation by a study of the rotations of DNP derivatives since a DNP amino acid usually has a much higher rotation than the free amino acid. Substitution on the δ-amino group of the ornithine or the imidazole of the histidine would not be expected to enhance the rotation. However, the approach proved to be complicated by the fact that bacitracin is transformed in part to the F type under the alkaline conditions of the DNP reactions. For this reason a rapid reaction was chosen in the earlier work[18] but in the present study the possibility of obtaining substitution at an acidic *p*H was explored. A *p*H of 4.66 was finally chosen. It was thought that a reaction time of 1 hr. at 40° would give mainly a mixture of the monoderivatives and the di-DNP derivatives with little substitution on the strongly basic delta amino group of the ornithine.

Three bacitracin preparations were studied in this way: a preparation of A from the 2-butanol/3% acetic acid system (low potency, $\alpha = -3.6°$), a preparation of A from run 3 (high potency, $\alpha = -2.4°$), and a sample of bacitracin B from run 4. After extraction with ether and dialysis to remove the buffer and unreacted bacitracin, the yellow

(17) L. C. Craig, J. D. Gregory and G. T. Barry, *J. Clin. Invest.*, XXVIII, 1014 (1949).

(18) L. C. Craig, W. Hausmann, and J. R. Weisiger, *J. Biol. Chem.*, 200, 765 (1953).

mixture was distributed in a system which gave about equal distribution of the color. All three bacitracin preparations gave remarkably similar patterns. Because of this only the pattern obtained with bacitracin B is shown, Fig. 2, in the interest of saving space.

Analysis of the band on the right at more than one wavelength indicated it to be a mixture and since it has too high a partition ratio to be separated most efficiently, this material was redistributed in a more favorable system as shown in Fig. 3. The different discrete bands from Fig. 2 and 3 are numbered in the order of their decreasing polarity. At least 10 substances are obviously formed by the reaction of FDNB on each bacitracin at *p*H 4.66.

In order to learn the nature of each band a sample was hydrolyzed and the hydrolysate was studied by paper chromatography using a variety of systems much as was done previously.[16]

In the case of the DNP reaction with bacitracin A from system 3 (the most potent preparation), band 2 was obviously the tri-DNP derivative since paper chromatography of the hydrolysate showed δ-DNP-ornithine, DNP-isoleucine, and the characteristic degradation products of im-DNP histidine. Free histidine and ornithine were lacking. The weight-optical density relationship at 350 mμ indicated[16] the molecular weight of the DNP derivative to be 1960 assuming three DNP groups, two of which give the molecular extinction found for di-DNP-histidine (14,500). Calculated: 1920.

A similar study of band 6 indicated the ornithine to be free. Otherwise it was the same as band 2 with no free histidine. The weight-extinction ratio indicated a molecular weight of 1730 again on the basis of the molecular extinction of di-DNP-histidine. Calculated for a di-DNP-derivative: 1754.

A similar study of band 9 showed DNP-isoleucine and all the free amino acids of bacitracin including the ornithine and histidine spots. Weight-optical density relationship at 350 mμ on the basis of 1 DNP permitted a value of 1810 to be derived. Calculated for acetate of mono DNP derivative, 1648.

Band 10 was not yellow and could be the mono-imidazole derivative while band 8 could be the mono-ornithyl derivative. The nature of bands 1, 3, 4, and 5 remain in doubt as yet. In 1 and 3 the ornithine, isoleucine, and histidine imidazole are covered. Bands 4 and 5 appear to be di-DNP derivatives with the ornithine and histidine imidazole being involved. One might expect to find three di-DNP derivatives, one known to be the DNP-Ileu, DNP-im of band 6, a DNP-Orn, DNP-im and a DNP-Orn, DNP-Ileu.

It is interesting to note that in the earlier work[18] on the partial substitution of A with the Sanger reagent at alkaline *pH* a good yield of a di-DNP band was obtained which had the imidazole of the histidine unsubstituted. Apparently this imidazole is more reactive to the Sanger reagent at an acid *p*H than at an alkaline one since there can be little of such a derivative in the mixture prepared here. Hydrolysis of all bands, 1 through 6, of Fig. 2 failed to show free histidine but showed the decomposition products of *im*-DNP-histidine.

When histidine itself was reacted with the Sanger reagent under the above conditions at *p*H 4.66 and the products distributed only one band was obtained. It proved to be the mono-alpha-DNP derivative. No di-DNP derivative was formed and very little, if any, mono-imidazole-DNP derivative. Under the alkaline conditions with $NaHCO_3$ two bands were obtained, one of which was the di-DNP derivative and the other the alpha-DNP derivative.

Sanger[19] found that α-acetyl histidine did not react readily with FDNB under the usual reaction conditions with $NaHCO_3$. Ramachandran and McConnell[20] found that when histidine reacts with insufficient FDNB under the same conditions only the alpha derivative is formed. With more reagent the di-derivative is formed but no mono-DNP-imidazole derivative. The reaction of FDNB with histidine is being further studied with the though that a transfer of the DNP group from the imidazole to the more basic nitrogen may be involved.

When the bacitracin A preparation of lower potency corresponding to the first two preparations of Table II was converted to the DNP derivative at *p*H 4.66 and studied in the same way as the high potency material the only definite difference noted thus far has been concerned with the optical activities of the bands. Table III shows the comparison of the rotations taken in glacial acetic acid although there was not sufficient material to take rotations with the highest precision as given in Table II. Nonetheless, these data strongly support the conclusion already reached from Table II that a loss in antibiotic potency is accompanied by a shift in the optical activity of at least one of the optically active centers. This center could be, and most likely is, in the terminal isoleucine but because this res-

TABLE III

OPTICAL ROTATIONS OF DNP DERIVATIVES $[\alpha]_D^{25}$

Band	DNP from High Potency Bacitracin A	DNP from Low Potency Bacitracin A	DNP from Bacitracin B
1	0	+38	− 4
2	−11	0	− 7
3	−17	0	0
4	−12	+26	−11
5	− 8	+10	..
6	−12	0	− 4
9	−16	..	− 9

(19) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(20) L. B. Ramachandran and W. B. McConnell, *Nature*, **176**, 931 (1955).

idue[2,3] may also be tied into the carboxyl of the phenylalanine residue in some obscure way, another residue also may be involved.

The behavior of bacitracin B has a bearing on this puzzling problem. Complete amino acid analysis by the Moore-Stein procedure gave the result shown in Table I. B differs from A by having an additional valine residue in it. However, ultimate analysis is in agreement with the formula, $C_{71}H_{112}O_{17}N_{18}S$, obtained by adding a valine residue to the formula of bacitracin A, only when an additional molecule of water is added. This, of course, may be a tightly bound water of hydration.

When bacitracin B was treated with the Sanger reagent under alkaline conditions a derivative was obtained after C.C.D. for which the weight-optical density relationship indicated a tri-DNP derivative. However, on total hydrolysis of this derivative paper chromatography clearly showed δ-DNP ornithine, the breakdown products of *im*-DNP-histidine, DNP-isoleucine, and, in addition, a spot in the position of DNP-valine. Free histidine and ornithine spots were lacking. All the other free amino acid spots expected were found, including valine.

In order to confirm the DNP-valine a distribution was made on the ether soluble fraction as given in the experimental part. DNP-valine was found in an amount corresponding to 33% of a mole as compared to 36% of a mole of isoleucine. The latter is a higher yield of DNP-isoleucine than has thus far been obtained[2] from tri-DNP-bacitracin A.

When bacitracin B was reacted with the Sanger reagent at *p*H 4.66, the results shown in Fig. 2 and 3 were obtained. Band 2 was the tri-DNP derivative already discussed above. M. W. from weight-optical density at 350 mμ, 1840; calculated, 1920. Hydrolysis and paper chromatography of band 6 indicated the ornithine to be free but the histidine to be involved. There was no spot of free histidine but only the spots characteristic of the acid degradation of *im*-DNP-histidine. Yellow spots corresponding to DNP-isoleucine and DNP-valine were found. Yet again the weight-optical density relationship at 350 mμ was consistent only with the presence of 1 DNP group in the intact DNP-peptide aside from the one on the imidazole, 1700; calculated, 1754.

Hydrolysis and paper chromatography of band 9 showed the ornithine and histidine to be free. Yellow spots corresponding to DNP-isoleucine and DNP-valine were found. Again the weight-optical density relationship with the intact DNP-peptide was consistent with only 1 DNP group, 1830; calculated for acetate of mono-DNP derivative, 1765.

Bacitracin B was found to be transformed to an F type even more rapidly than A. The F from B on complete hydrolysis showed a strong valine spot with paper chromatography. Since it is known[2] that F from A is formed by loss of the amino group of the terminal isoleucine,[21] the valine cannot be terminal in bacitracin B unless it is attached in some manner in the vicinity of the isoleucine so that isomerization is possible and either the isoleucine or valine, but not both, could serve as the terminal group. The latter possibility could explain the behavior found with the DNP derivatives.

Even with respect to the DNP derivatives of bacitracin A it could be that the postulated linkage from the so-called terminal isoleucine to the carboxyl of the phenylalanine could make the α-amino group of the histidine reactive to the DNP reagent. Here it would be a case of the DNP group becoming attached to either the isoleucine or the α-amino of the histidine, but not both. Its presence on the α-amino of the histidine could not be detected since the histidine DNP derivatives decompose on hydrolysis. This theory would explain the anomalously low yield of DNP-isoleucine obtained on hydrolysis of tri-DNP-bacitracin A.[2]

Two other explanations for the behavior of the bacitracin polypeptides should not be overlooked. One concerns the possibility that the DNP group can be transferred from one position to another during acid hydrolysis. In this connection the histidine and thiazoline ring could be suspect.

The other could be based on the theory that in spite of all the precise fractionation thus far done on the free peptides and DNP derivatives, the preparations are still mixtures of closely related peptides. The data presented in this paper indeed do seem to indicate that they may not be entirely pure with respect to the optical configurations of all the amino acids. Similarly, the data are not sufficient to exclude mixtures of the type where in one an isoleucine and a valine residue have exchanged places. If so, this could indicate a certain lack of specificity in the enzyme systems which have produced the peptides. In the particular case above this could be investigated by isolation of the ketothiazole[2] from F prepared from bacitracin B to determine if any of it has the isobutyryl side chain. This work is in progress.

The alloisoleucine formed (0.5 mole) on hydrolysis of A comes from the terminal isoleucine since tri-DNP-bacitracin A and F from A do not give a detectable amount.[2] This indicates complete racemization of the alpha carbon atom of the isoleucine during hydrolysis. Bacitracin B gave only 0.330 mole (0.355 mole on a check analysis). Abraham[22] obtained no alloisoleucine when hydrolysis of A was preceded by deamination. Also the isoleucinol formed on hydrogenolysis of A gave 33%

(21) The ketothiazole[2] postulated on hydrolysis of F has been synthesized in this laboratory and will soon be described by Dr. J. R. Weisiger.

(22) E. P. Abraham, 3rd Congr. Intern. Biochem., Brussels, 1955, 423.

allo when oxidized back to isoleucine. A recent analysis of bacitracin A of high potency from system 3 again gave 0.5 mole of alloisoleucine.

Acknowledgment. We wish to thank Mr. James Rigakos for the ultimate analyses reported in this paper. Thanks are also due Miss Gerty Walker and Mrs. Judy O'Brien for technical assistance.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE COBB CHEMICAL LABORATORY, UNIVERSITY OF VIRGINIA]

Pinacollike Rearrangement of a Cyclopropane-1,2-dimethylene Glycol*

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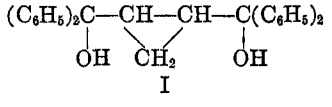
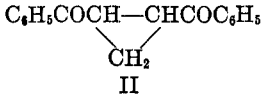
The cyclopropylog[2] of a 1,2-glycol, namely *trans*-1,2-di(diphenylhydroxymethyl)cyclopropane, was made by addition of phenyllithium to *trans*-1,2-dibenzoylcyclopropane. It underwent facile acid-catalyzed pinacol-type rearrangement with shift of a phenyl group to the adjacent cyclopropane carbon and concomitant cleavage of the ring, to produce the unsaturated ketone, 1,2,5,5-tetraphenyl-4-pentene-1-one. The structure of this product was demonstrated by its properties and by oxidative degradation to β-benzoyl-β-phenylpropionic acid.

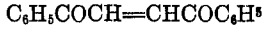
The rearrangement of the cyclopropylog[2] of a 1,2-glycol, namely *trans*-1,2-di(diphenylhydroxymethyl)cyclopropane (I), was discovered in the course of new studies on the preparation and reactions of *trans*-1,2-dibenzoylcyclopropane (II)[3,4] which had been undertaken for the purpose of comparing the conjugated system of this latter compound (II) with that of the olefinic analog, *trans*-1,2-dibenzoylethylene (III).



I

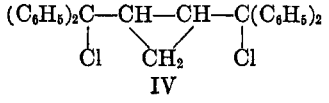


II

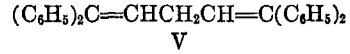


III

The cyclopropane-1,2-dimethylene glycol (I) was made by the action of phenylmagnesium bromide or phenyllithium on 1,2-dibenzoylcyclopropane (II). It had been the authors' intention to subject both this glycol (I) and the corresponding dichloride (IV) to the action of reducing agents. It was believed that possibly the conjugation between the functional groups through the cyclopropane ring might to some extent permit or favor 1,6-reduction with consequent formation of 1,1,5,5-tetraphenyl-1,4-pentadiene (V), reactions for which there are ample olefinic analogies (*cf.* the 1,6-reduction of pseudocodeine[5,6] and the 1,4-reductive-elimination of halogens from 2-butene-1,4-dihalides[7]).

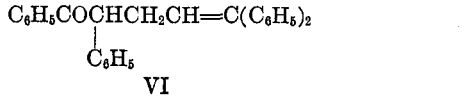


IV



V

In an attempt to prepare the dichloride (IV), the glycol (I) was subjected to the action of thionyl chloride. The crystalline product, however, proved to be halogen-free and analysis and molecular weight established the empirical formula $C_{29}H_{24}O$. The infrared absorption spectrum contained a band corresponding to a benzoyl-type carbonyl group (5.94 μ), and did not have the characteristic strong cyclopropane band in the 9.8–10 μ region. These results led to the conclusion that there had occurred a pinacol-type rearrangement with the glycol (I) functioning in the sense of a cyclopropylog[2] of a tetraphenyl-1,2-glycol, and that the product was the pinacolone partial-analog (VI).



VI

Consistent with the above formulation of the product (VI) the ultraviolet absorption spectrum of the compound showed a strong band at 248.5 mμ, ε, 16,530, which is significantly close to the summation of the expected molar absorptivities of the two independent chromophores, benzoyl and

* This paper is a contribution in honor of Lyndon F. Small, former Editor of the Journal.

(1) Philip Francis du Pont Fellow, 1955–57. Assistance during the summer from an Office of Ordnance Research Contract, U. S. Army, is acknowledged. Present location, Experimental Station, E. I. du Pont de Nemours, Inc., Wilmington, Del.

(2) We use this term in a sense comparable with the terms homolog and vinylog.

(3) R. A. Darby, dissertation, University of Virginia, May 1957. The cyclopropanes I and II were assigned *trans* configurations on the basis of failure of II to isomerize under the action of 1% alcoholic sodium hydroxide, conditions which readily effect rearrangement of the unstable stereoisomer of 3-phenyl-1,2-dibenzoylcyclopropane. (See ref. 15).

(4) J. B. Conant and R. E. Lutz, *J. Am. Chem. Soc.*, **49**, 1083 (1927).

(5) R. E. Lutz, *J. Am. Chem. Soc.*, **56**, 1378 (1934).

(6) R. E. Lutz and L. F. Small, *J. Am. Chem. Soc.*, **56**, 2466 (1934).

(7) J. Thiele, *Ann.*, **308**, 339 (1899).